Why two kinds of stamens in buzz-pollinated flowers?
Experimental support for Darwin’s division-of-labour hypothesis

Z. Luo,1 D. Zhang1* and S. S. Renner2

1South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou 510650, China; and2Department of Biology, University of Munich, D-80638 Munich, Germany

Summary

1. Most animal-pollinated flowering plants offer nectar as a reward for their pollinators. Some 20 000 species, however, offer only pollen and rely on pollen-foraging bees for pollination. This creates a dilemma since pollen grains contain the male gametes and should be protected from becoming bee food. Darwin was the first to hypothesize that a ‘division-of-labour’ among stamens could solve this dilemma, with some stamens providing pollen as food, others providing pollen for fertilization. This hypothesis can only be tested if pollen grains from the two sets of stamens can be distinguished and their fates determined to the point of attachment on the stigma.

2. We tested Darwin’s hypothesis in Melastoma malabathricum (Melastomataceae), a pollen-only flower with conspicuously differentiated stamens, an inner set that is short and yellow and an outer set that is much longer and purple with small yellow spurs. Pollen release is through terminal anther pores. Taking advantage of different exine patterns on the pollen produced by the two sets of stamens, we carried out a series of experimental manipulations to compare pollinator foraging behaviour and pollen pathways from anthers to stigmas.

3. The results demonstrate that in spite of all 10 stamens being buzzed simultaneously by the carpenter bees that pollinate M. malabathricum, pollen from the purple ‘fertilization’ stamens is dramatically more likely to land on stigmas than pollen from the yellow ‘feeding’ stamens. Removal experiments showed that the yellow ‘feeding’ stamens also attracted pollinators from a distance. Flowers that had their anther pores plugged received fewer buzzing bouts per visit, indicating that bees assessed the amount of pollen received per bout. Surprisingly, there was no significant difference in pollen loads on stigmas of flowers that had their anthers plugged and stigmas of controls, demonstrating the efficiency of vector-assisted cross-pollination and the lack of vector-assisted self-pollination.

4. The unexpectedly precise placement of pollen grains even with buzz pollination, with a large proportion of the grains deposited out of the bees’ grooming reach, helps explain the evolutionary persistence of pollen as a reward in spite of the bees’ ability to assess the amount of pollen received during foraging bouts. Together, these results strongly support Darwin’s division-of-labour hypothesis.

Key-words: bee foraging, buzz pollination, Melastoma, pollen dimorphism, pollination efficiency, pollinator attraction, stamen dimorphism

Introduction

Some 20 000 species of flowering plants offer only pollen as a reward for their pollinators and are adapted to pollination by bees that depend on pollen to feed their larvae (Buchmann 1983; Michener 2007). This creates a dilemma because pollen grains contain the male gametes and plants therefore need to protect them from bees. A widespread feature of such pollen-offering flowers is stamen dimorphism, involving two sets of stamens that differ in position, size, colour, and/or shape (Buchmann 1983). To explain this phenomenon, Darwin (1862) hypothesized that it reflects a ‘division-of-labour’ among stamens. One set of stamens would satisfy the pollinators’ demand for pollen as food (the ‘feeding’ stamens), the other would satisfy the plant’s need for safe gamete transport.
of the flower, guided by gravity (Troll 1922), resulting in a zygomorphic androecium. The stamens of the outer whorl have pale purple anther sacs (11–15 mm long) and small yellow connective spurs (c. 3 mm long); their filaments are 10–16 mm long and yellowish-white. When the petals first unfold at sun rise, the outer stamens move to the lower part of the flower (Fig. 1a). According to the division-of-labour hypothesis, the five yellow inner stamens function predominantly as feeding stamens (FS), while the five pupillish outer stamens function in fertilization (here we refer to them as pollination stamens, or PS). Experimental selfing confirmed earlier findings that *M. malabathricum* (synonym *M. affine*) is self-compatible (Gross 1993).

Most Melastomataceae have poricidal anthers and are pollinated by bees capable of collecting pollen through sonicating the anthers with the help of their indirect flight muscles (Buchmann 1983; Renner 1989; King, Buchmann & Spangler 1996). In such ‘buzz’ pollination, bees usually directly alight on the stamens, grasp them firmly with their legs, and then apply high frequency vibrations that expulse pollen grains from the anther pores.

Observations and experiments were carried out from 20 June to 20 August in 2005 and 2006, and from 15 June to 15 September in 2007 at the South China Botanical Garden (SCBG), a 300-ha nature reserve within the city area of Guangzhou, in Huolushan Forest Park, and in the Dinghushan Nature Reserve, all in Guangdong province. A voucher (Luo 24) has been deposited in the herbarium of the South China Botanical Garden (official acronym IBSC).

**Experimental design**

Quantification of pollen and ovules

To test if there were differences in the amount of pollen produced by the two sets of stamen, 10 flower buds from five individuals were collected 1–2 days prior to anthesis and stored in formalin-acetic acid–alcohol in a refrigerator. One pollination anther and one feeding anther per bud were dissected and crushed separately in 1 mL staining solution (a 1 : 3 mixture of glycerine/aniline-blue in lactophenol). The suspension was stirred with a vortex mixer for 60 s, and 10 separate samples of 1 μL each were then transferred onto slides with an Eppendorf transferpettor (Eppendorf, Germany). Pollen grains on each slide were counted under a microscope (Olympus BX-41; Olympus, Japan) and the numbers then multiplied by the dilution factor (1000) to obtain the total number of pollen per anther. Ovary locules of 10 flowers were dissected under a stereoscope (Stemi DV4; Zeiss, Germany) and the ovules counted at 40× magnification.

Pollen viability and stigma receptivity

We used triazolyl blue tetrazolium bromide (MTT) to test for the presence of dehydrogenase in pollen as an assay for pollen viability.
Ten flowers from five individuals were netted just before anthesis to prevent insect visits; pollen viability in one PS and one FS was then tested every three to four hours from anthesis to flower wilting. The same assay was used to assess stigma receptivity (Rodriguez-Riaño & Dafni 2000), using the same numbers of experimental plants. All tests were carried out on warm, sunny days.

**Pollen morphology**

Pollen morphology was observed and photographed using a Scanning Electron Microscope (SEM, type JSM-6360LV, JEOL, Japan). Pollen grains were thin-sectioned with a Leica EM UC6 ultramicrotome (LEICA, Germany) and then stained with uranyl acetate and lead citrate. Thin sections were examined and imaged using a Transmission Electron Microscope (TEM, type JSM-1010, JEOL, Japan).

**Pollinator behaviour on the two types of stamens**

On some 20 sunny days between 07.30 and 17.30 h in July and August 2005, 2006, and 2007, flower visitors were recorded, photographed, or collected for later identification. Special attention was paid to whether visitors contacted the stigmas. Vouchers of the bee species have been deposited in the collection of the Zoologische Staatssammlung Munich and in the personal collection of D. Zhang.

To test the visual (and perhaps also olfactory) attractiveness of the two sets of stamens to floral visitors, we carried out experiments in a population of c. 15 shrubs at SCBG. Treatments were performed on five sunny days in 2007 when *M. malabathricum* was at peak flowering. Sets of 20 flowers were manipulated as follows: a) flowers left intact (Control); b) pollination stamens removed (FS); c) feeding stamens removed (PS); d) feeding stamens and anthers of pollination stamens removed, leaving the filaments and yellow connective spurs of the PS intact (PS fia); e) all stamens completely removed (NS).

To test whether bees were able to assess the amount of pollen received per visit, we manipulated an additional set of flowers (FS bl) by cutting the pollination stamens off and plugging the anther pores of the feeding stamens with a minute amount of an inert glue (an odourless adhesive similar to Elmer’s glue; Buchmann & Cane 1989) so that flowers no longer offered any reward, but remained visually attractive because of the yellow colour of the plugged feeding stamens. Following Buchmann & Cane (1989), we applied the same amount of glue to the anther sac surfaces of 20 control flowers as an odour control.

Treatments were repeated with fresh flowers on each of five days, and *Xylocopa* visits to each set of manipulated flowers were recorded. Buzzing bouts per visit were counted using the audible sonicating behaviour employed by the bees to expel pollen grains from the anthers, and at least 60 visits or approaches were recorded.

**Vector-assisted cross-pollination vs. vector-assisted self-pollination**

Forty flower buds on four individuals were bagged one day before anthesis. At anthesis, the anther pores in half the flowers were plugged (or blocked) with a minute amount of glue to prevent the stigmas from being contaminated with pollen from the same flower during a buzzing bout. Stigmas were collected when a flower had been buzzed once. To examine the extent of vector-assisted self- or cross-pollination, pollen grains on each stigma from the two treatments were counted under a microscope.

**Placement of pollen grains from the two types of stamens on bees and on stigmas**

Ten *Xylocopa* bees that had visited *M. malabathricum* flowers were captured, dissected, and examined under a SEM. The proportions of pollen grains from the pollination and feeding stamens (which could be distinguished because of their different exine ornamentation; Results) on the dorsal and lower ventral surface of each bee’s abdomen and the ventral side of its thorax were calculated. At least 200 grains were counted on each insect.

Stigmas of virgin flowers (flowers bagged before anthesis) that had been exposed to a single visit by a *Xylocopa* bee in the natural habitat (i.e., a bee carrying *M. malabathricum* pollen collected during normal foraging bouts with natural grooming) were collected, fixed, and examined under the SEM. Several randomly chosen areas on each stigma were examined and ‘fertilization’ and ‘feeding’ pollen grains counted to calculate the proportions of each type. A sufficiently large surface was examined to count at least 200 grains stigma⁻¹.

Fluorescent powder (Guangzhou Research Institute of Nonferrous Metals, Guangzhou, China) was used as a marker to examine the place of contact between stigmas and the bees’ bodies. In the morning, stigmas of 24 freshly opened flowers on three plants were marked with fluorescent powder. Bees were captured after they had visited marked flowers and examined under UV light with the aid of a stereoscope.

**Statistical analyses**

Student’s *t*-tests were used to compare the differences in pollen number between the two sets of stamen and the proportions of ‘fertilization’ and ‘feeding’ pollen on stigmas and bees’ bodies. One-way ANOVA was used to test for differences in pollinator visiting frequency and bees’ buzzing rates among experimental flower treatments. Data of percentages were arcsine transformed before analyses, as they are not normally distributed. All statistical analyses were performed using spss version 13.0.

**Results**

**Differences between the pollen grains from the two types of stamens**

Pollination stamens contained significantly more pollen grains than did feeding stamens (233 106 ± 15 562 vs. 167 388 ± 7305 per stamen, mean ± SE, *t* = 6·97, *P* < 0·001, *N* = 10). An average flower produced 1 165 530 ± 77 810 grains in its fertilization anthers and 836 940 ± 36 525 grains in its feeding anthers. An average ovary (*N* = 10) contained 2172·9 ± 80·2 ovules. The pollen/ovule ratio therefore was 922·3 ± 38·78.

The percentage of viable pollen grains in the PS was significantly higher than that in the FS during the 1-day flowering period; percentages were about 83·7 ± 0·33% vs. 41·3 ± 3·3% (mean ± SE) at flower opening and 39·6 ± 7·4% vs. 29·5 ± 1·8% when flowers began to wilt. Stigmas remained receptive from anthesis to wilting.

As is typical of Melastomataceae, pollen grains of *M. malabathricum* are shed singly, and are spherical and three-colporate. Under the SEM, the exine ornamentation of pollen from...
Support for Darwin’s division-of-labour hypothesis

the PS and the FS differs, with the PS pollen being micro-rugulate (Fig. 1b), the FS pollen striate or micro-striate (Fig. 1d). In TEM, the tectum of feeding pollen is coarser than that of fertilization pollen, with the space between the columellae arranged more irregularly (Fig. 1c,e).

POLLINATOR BEHAVIOUR ON THE TWO TYPES OF STAMEN

At our study sites, M. malabathricum flowers were pollinated by Xylocopa collaris Lep. forma alboxantha Maa and X. nasalis Westwood. The two species visited the flowers frequently, behaved similarly, and were the only visitors that touched the stigmas while buzzing the stamens to collect pollen. Xylocopa usually alighted on the feeding stamens, grasped their five anthers together with the connectives of the fertilization stamens, and buzzed the entire set for 2–3 s ($N = 129$, SCBG, 2006). They also milked the feeding anthers with their mandibles, leaving dark necrotic marks on anthers that had been buzzed several times. During buzzing bouts, the tips of the pollination stamens touched the bees’ sides and abdomens, while the tips of the feeding stamens were placed close to the bees’ mouthparts. In bright sunlight, clouds of pollen grains could be seen being emitted from the anther pores of the PS and settling on the ventral and dorsal abdomen, which is the part of the bee body that will touch the stigma during a visit to the next flower.

Less common visitors were Amegilla zonata bees, which due to their small body size buzzed one stamen at a time and rarely touched the stigmas. Trigona bees, which extracted pollen with their mandibles, acted as pollen robbers as observed in many other Melastomataceae (cf. Renner 1983). Syrphid flies occasionally scavenged pollen grains from the petals.

The visiting frequencies of Xylocopa bees varied significantly among different experimental flower treatments ($F = 74·13$, $P < 0·001$; Fig. 2). Flowers that had the pollination stamens removed ($FS$ and $FS$ bl) were visited as frequently as un-manipulated (Control) flowers ($P = 0·46$, $FS$ vs. Control; $P = 0·84$, $FS$ bl vs. Control). There was no significant difference in Xylocopa visiting behaviour between flowers that had received glue on their anther surfaces and those that had not (Control), showing that the glue did not influence visitor behaviour.
When the feeding stamens were removed (PS), visitation declined sharply ($P < 0.001$). During visits to such flowers, Xylocopa bees landed on the (purplish) pollination stamens and then vibrated their yellow connective spurs once (rarely twice), after which they would depart quickly. Visiting frequencies between flower sets PS and PS fila were similar ($P = 0.45$; Fig. 2), demonstrating that the purplish anthers of the pollination stamens had little visual or olfactory attraction to the carpenter bees (both flower sets retained the yellow connective spurs of the pollination anthers). Flowers that had all stamens completely removed were approached by Xylocopa, but bees did not alight on them.

Flowers with the pollination stamens removed, but the feeding stamens present and unplugged (FS) experienced as many buzzing bouts as intact flowers (Control) ($P = 0.18$, FS vs. Control). Flowers with the pollination stamens removed and the pores of the feeding stamens plugged (FS bl), however, experienced many fewer buzzes per visit ($P < 0.001$, FS bl vs. Control). Numbers of buzzing bouts per visit also differed significantly between experimental treatments ($F = 91.66$, $P < 0.001$; Fig. 3).

VECTOR-ASSISTED CROSS-POLLINATION VS. VECTOR-ASSISTED SELF-POLLINATION

Stigma pollen loads were large (~1000, $N = 40$), even after a single visit by a Xylocopa. There was no significant difference in pollen loads on stigmas of flowers that had their anthers plugged and stigmas of controls (1104.6 ± 118.1 vs. 1228.6 ± 88.3 grains, $t = 0.84$, $P = 0.41$, $N = 20$), demonstrating the efficiency of vector-assisted cross-pollination and the lack of vector-assisted self-pollination. The average duration of bees’ first visits also did not differ between flowers with plugged anthers and controls (1.86 ± 0.11 s vs. 1.69 ± 0.10 s, $t = 1.18$, $P = 0.24$, $N = 40$). Bees, however, rarely stay for a second bout on an anther-plugged flower. Since the average ovary contains 2173 ovules, a single visit provides about 0.56 pollen grains/ovule, and thus at least two visits are needed to provide sufficient grains to assure reasonable seed set in any one flower.

PLACEMENT OF POLLEN GRAINS FROM THE TWO TYPES OF STAMENS ON BEES AND ON STIGMAS

Because the ‘fertilization’ and ‘feeding’ pollen grains had different exine ornamentation, they could easily be distinguished on stigmas under an SEM. Stigmas carried significantly more fertilization than feeding pollen (82.2 ± 6.42% vs. 17.8 ± 6.42%, $t = 58.5$, $P < 0.001$, $N = 24$). On the bees, most fertilization pollen was found on the dorsal and lower ventral abdominal (91.0 ± 0.10% fertilization pollen, $t = 29.4$, $P < 0.001$, $N = 10$), the part that touches the stigma, while most feeding pollen was found on the ventral side of thorax (80.4 ± 1.56% feeding pollen, $t = 16.7$, $P < 0.001$, $N = 10$; Fig. 4).

Seven Xylocopa bees were captured on flowers whose stigmas had been labelled with fluorescent powder. Under UV light, fluorescent powder particles were found on the dorsal or lower ventral surfaces of their abdomens.

Discussion

Our data provide the first unequivocal support for Darwin’s division-of-labour hypothesis explaining stamen dimorphism in buzz-pollinated flowers that offer only pollen as a reward. In flowers that offer a nectar reward, functional specialization between different sets of stamens can lead to deceptive stamens that contain little or no pollen (e.g., Percival 1965;
Vogel 1978). In buzz-pollinated flowers, however, selection from bees places a lower limit on the pollen provisioning needed to retain pollinator interest. This was experimentally demonstrated by the comparison of bees’ foraging efforts in anther-plugged and un-manipulated flowers. Buzzing bouts per visit declined by about 50% when feeding anthers were plugged, despite similar visiting rates. The reason was that carpenter bees rarely buzzed plugged (hence unrewarding) anthers for a second time, in agreement with studies showing that bees assess pollen quantities received during sonication (Buchmann & Cane 1989; Harder 1990). Visiting rates would decline sharply, however, when flowers lacked the feeding stamens, demonstrating that their bright yellow colour (and perhaps also smell) was attracting bees. A guide function of yellow stamens or staminodes has also been demonstrated in other studies (Lunau 2000; Ushimaru, Watanabe & Nakata 2007). The similar visiting frequencies of Xylocopa to flowers with and without pollination anthers (treatments PS vs. PS/fila) demonstrated that the purplish anther sacs of the pollination stamens were not attracting bees.

Colour dimorphism within androecia is relatively common and can have multiple functions, including deception of visitors by brightly coloured, but rewardless decoys (Pericival 1965; Mattsson 1976; Vogel 1978; Bahadur, Chaturvedi & Swamy 1990; Hrycan & Davis 2005). In M. malabathricum, however, both sets of stamens must contain pollen and must be buzzed for a flower to fulfil its function: The feeding anthers need to provide enough pollen to attract at least two visits by a Xylocopa for the stigma to receive sufficient grains to pollinate all ovules of an ovary, and the fertilization anthers also need to be buzzed for the flower to export its gametes (which would not occur unless the feeding stamens provided an award). The key to the successful division-of-labour among the two sets of stamens therefore is not differential visual attractiveness of the stamens, but the small diameter of the anther pores through which pollen grains are ejected in precisely pointed streams onto particular body sections of the large bees that are the effective pollinators of M. malabathricum (van der Pijl 1939, 1954; Gross 1993; Momose et al. 1998; personal observation; this study). In this way, the feeding stamens release their grains towards the bees’ mouthparts and thorax, while the fertilizing stamens release theirs onto parts of the bees’ bodies that are least accessible during pollen grooming (Michener 2007) and that come into close contact with the stigma. Extremely predictable pollen placement fits with the observed low P : O ratio (Cruden 1977; Cruden & Miller-Ward 1981). It would also fit with selfing, but self-pollination is not a large factor as revealed by the experiments that compared pollen deposition on the stigmas of anther-plugged flowers with un-manipulated controls and which found almost the same number of pollen grains present after a single bee visit. This demonstrates the extreme efficiency of cross-pollination and the near lack of vector-assisted self-pollination in M. malabathricum.

Because both sets of stamens must be buzzed for the flowers to function, and as long as feeding pollen occasionally ends up on stigmas, selection should favour the retention of pollen viability in both sets of stamens. Contrasting reports on pollen viability in M. malabathricum probably result from the vagaries of the pollen viability assays used and perhaps also from different times at which the pollen was collected or tested. Thus, Percival (1965) and this study found viability differences between pollen from the two sets of stamens, while Gross & Kukuk (2001) did not.

Stamen dimorphism of the kind found in Melastoma and other clades of Melastomataceae (Clausing & Renner 2001) effectively solves the dilemma faced by buzz-pollinated flowers between the need to satisfy pollen-seeking bees and the need to safeguard gametes from consumption. Rapid learning and facultative responses by bees can complicate the picture, however. When small bees that only buzz one or a few stamens (Thorp & Estes 1975; Renner 1989) visit large flowers, such as those of M. malabathricum, they can learn to preferentially empty the longest anthers first (Gross & Kukuk 2001), thus acting as pollen thieves. This was true of Amegilla anomola (on M. malabathricum in Australia; Gross & Kukuk 2001) and of A. zonata (this study). Similar complex interactions between bee size and stamen size probably underlie the repeated evolution and loss of stamen dimorphism in the tens of thousands of angiosperms with buzz-pollinated flowers. In the genus Melastoma, stamen dimorphism is ancestral and was lost in a few small-flowered species in which all 10 stamens are positioned in a circle around the stigma (Meyer 2001; Renner & Meyer 2001). Only flowers capable of subdividing the body surfaces of relatively large pollinators can harness the potential precision of pollen placement to protect their gametes from ending-up in bees’ collecting apparatuses.

References


van Nostrand Reinhold, New York.


Received 27 February 2008; accepted 4 June 2008

Handling Editor: Gaku Kudo